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Terminal Deoxynucleotidyl Transferase in a Case of Childhood Acute Lymphoblastic Leukemia

(thymocytes/DNA polymerases)

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ABSTRACT **Cells from a patient with childhood acute lymphoblastic leukemia contain an apparent DNA polymerase activity that was not found in any other cells except thymus cells. The enzyme has the properties of terminal transferase, an enzyme known to be found in thymocytes. The cells also contain the three major DNA polymerases found in growing cells. The results suggest that these tumor cells arose from a block in the differentiation of thymocytes. Terminal transferase may be a marker for the origin of leukemic cells.**

Mammalian cells contain several DNA polymerases (1–8). We have been analyzing the content of soluble DNA polymerases in various types of cells, using phosphocellulose columns to separate the activities from a crude extract, and a series of synthetic template·primer combinations to help identify the enzymes. Using this methodology, we have recently observed in cells of a child with acute lymphoblastic leukemia (ALL) a DNA polymerizing activity that was not present in many other types of cells. This paper describes the observation and the evidence that this enzyme is terminal transferase, an enzyme specific to thymocytes (9).

The nomenclature of mammalian DNA polymerases is confusing, since a wide range of inconsistent sets of names is used for the enzymes. From both the literature and our own analyses, there appear to be three major DNA polymerases in most mammalian cells. As a necessary background to the presentation of our work we have developed the following nomenclature. The 6-8S DNA polymerase, found mainly in the cytoplasmic portion of cell extracts, is DNA polymerase C (1, 10, 11). The content of this enzyme in cells varies widely and is dependent on the growth rate of the cells (Chang, McKay, & Bollum, *J. Mol. Biol.*, in press). The second enzyme, which sediments at 3.3 S, is called DNA polymerase N, and is generally recovered from the nuclear fraction of cell extracts (3, 5). This enzyme is detected in appreciable amounts in all cells and does not vary with the rate of cell growth (3, 5, 12). A similar activity, present in the cytoplasm, is assumed to be related to the nuclear enzyme. The third enzyme, assayed by measuring poly(dT) synthesis stimulated by poly(A)·oligo(dT) in the presence of Mn^{++} (6, 13), is called DNA polymerase A. It is present in most cells that we have examined, and has been reported by many investigators (13–15). It has been considered to be a "reverse transcriptase in uninfected cells," but there seems to be no utility in this designation.

Abbreviation: ALL, acute lymphoblastic leukemia.

MATERIALS AND METHODS

Cells

Acute Lymphoblastic Leukemia. Venous blood was obtained by phlebotomy from a 5.5-year-old child with acute lymphoblastic leukemia. At the time of phlebotomy the peripheral leukocyte count was 600,000/mm^3; 99% of these cells were morphologically typical acute lymphoblastic leukemia cells. Bone marrow aspiration disclosed total replacement of normal marrow elements by cells similar to those in the blood. Adenopathy and splenomegaly were present. Chest x-ray showed no thymic enlargement. The cells were harvested from blood anticoagulated with acid-citrate dextrose and mixed with 25% by volume of 6% dextran; the erythrocytes sedimented at 37° for 1 hr. The lymphocytes were then collected from the supernatant plasma by low-speed centrifugation.

Other Leukemias. Cells were obtained in the same manner from a 59-year-old man with lymphosarcoma cell leukemia (leukocyte count, 120,000/mm^3; 98% lymphosarcoma cells) and from a 10-year-old child with acute myeloblastic leukemia (leukocyte count, 120,000/mm^3; 95% leukemia cells, several with Auer rods).

HeLa Cells. 1 Liter of HeLa cells at 4×10^5/ml, grown as described (16), were harvested by centrifugation and washed three times with 10 volumes of Earle's saline (17).

Cultured Human Lymphocytes. Two lines of cultured human lymphocytes were used. One line, designated L33-6-1-19, was derived from line PGLC 33H, initiated about 5 years ago by Dr. P. R. Glade from a patient with infectious mononucleosis and was kindly provided by Dr. H. Ronald Zielke. The second line, CCRF-CEM, was initiated from a patient with acute lymphoblastic leukemia in 1965. About 10^8 cells of each line were processed after extensive washing with Earle's saline.

Thymus. A 15-g specimen of thymus from a hematologically and immunologically normal 5-month-old child (removed to facilitate exposure during cardiac surgery) was supplied by Dr. Judah Folkman. The tissue was homogenized in a Waring Blendor at low setting for 5 min in the presence of 125 ml of TEM buffer [0.05 M Tris·HCl (pH 7.7)–1 mM EDTA–1 mM 2-mercaptoethanol] at 4°, and thereafter processed in a manner identical to the crude homogenates of other cells as described below.

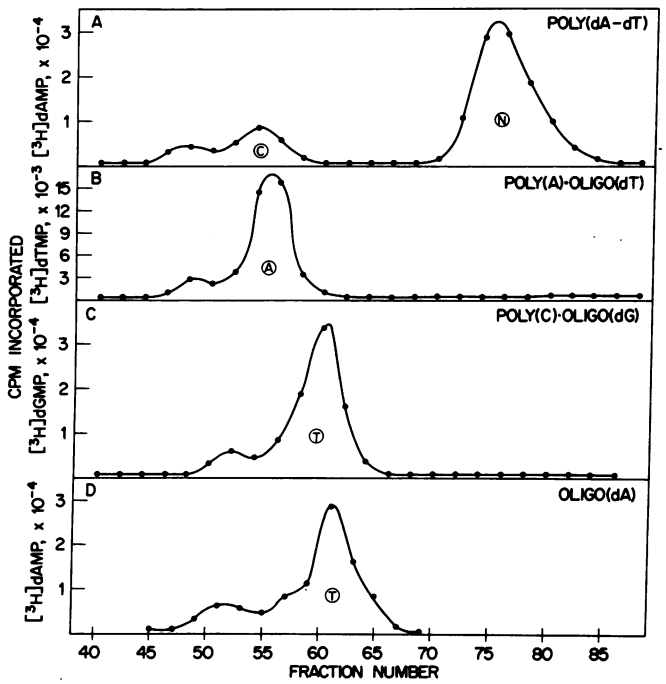

FIG. 1. DNA polymerases of cells from a patient with acute lymphoblastic leukemia. The individual panels represent assays of the fractions from a single phosphocellulose column with various templates, primers, and substrates as indicated. Standard reaction conditions were used for each set of assays in panels *A*, *B*, and *C*. For panel *D*, 12,000 pmol of oligo(dA) plus 1200 pmol of [³H]dATP (1600 cpm/pmol) were used with 0.8 mM $MnCl_2$. The *circled letters* indicate the peaks corresponding to DNA polymerases C, N, A, and T, as defined in the text.

Analysis procedure

The purification procedure used was modified from that developed by Mantsavinos (18). All operations were performed at 0°–4°.

Crude Extract. Cells were washed repeatedly in Earle's saline and resuspended in TEM buffer. The resuspended cells were subjected to three cycles of rapid freezing and thawing, Triton X-100 was added to a final concentration of 0.5%, and the homogenate was mixed thoroughly. The detergent-treated crude extract was centrifuged at 144,000 × *g* for 60 min and the supernatant fluid was saved.

pH 5.0 Precipitate. The pH of the high-speed supernatant was reduced to pH 5.0 by the dropwise addition of 1 M acetic acid, and stirred for 15 min. The turbid suspension was centrifuged at 15,000 × *g* for 10 min and the supernatant was discarded. The pH 5.0 precipitate was extracted by gentle homogenization in TEMG buffer (TEM plus 20% w/v glycerol). Insoluble material was removed by centrifugation at 15,000 × *g* for 10 min, and the supernatant was applied directly to a phosphocellulose column.

Phosphocellulose Chromatography. Phosphocellulose, Whatman P11, was prepared by the method of Burgess (19). The resin was equilibrated with TEMG buffer and a column, 0.5 × 12 cm, was prepared. In a typical purification the redissolved pH 5.0 precipitate (5 ml; 2 mg of protein per ml) from 3 × 10^8 cells starting material was applied to the column at a flow rate of 0.2 ml/min, and this flow rate was maintained.

After a 5-ml wash with TEMG buffer containing 1 mg/ml of bovine-serum albumin, the column was eluted with a 60-ml linear gradient of 0–1 M KCl in TEMG buffer containing 1 mg/ml of bovine-serum albumin; fractions of 0.7 ml were collected.

Assays of DNA polymerase activity

All reactions were performed at 37° for 1 hr in 0.1-ml volumes in the presence of 0.05 M Tris·HCl (pH 8.3)–6 mM dithiothreitol. Reaction mixtures contained 50-μl portions of the phosphocellulose fractions. Templates, primers, and deoxynucleoside triphosphates were obtained from commercial sources (20). Standard reaction mixtures contained one of the following sets of reagents (concentrations are given as monomer in a polymer):

Poly(dA-dT) Reaction: poly(dA–dT) 30 nmol; $Mg(acetate)_2$, 6 mM; [³H]dATP, 1200 pmol, 1600 cpm/pmol; dTTP, 2000 pmol.

Poly(A)·Oligo(dT) Reaction: poly(A), 3000 pmol; dT_{12-18}, 1500 pmol; $MnCl_2$, 0.8 mM; [³H]dTTP, 1000 pmol, 2000 cpm/pmol.

Poly(C)·Oligo(dG) Reaction: poly(C), 2700 pmol; dG_{12-18}, 1350 pmol; $MnCl_2$, 0.8 mM; [³H]dGTP, 1780 pmol, 2000 cpm/pmol.

RESULTS

DNA polymerases in ALL cells

A soluble extract of the tumor cell population from the blood of an ALL patient was assayed for its content of DNA polymerases. The three normal DNA polymerases were evident in a KCl gradient eluate from the phosphocellulose column (Fig. 1). With poly(dA-dT) as a template·primer, two enzymes were detected (Fig. 1*A*). The first to elute, at 0.28 M KCl, was DNA polymerase C, whose activity was demonstrable only in small amounts. (This enzyme, like all the detectable enzymes in this preparation except DNA polymerase N, had a minor fraction eluting before the major peak.

TABLE 1. *Dependence of dGMP incorporation on poly(C) and oligo(dG)*

Additions	Avian myeloblastosis virus DNA polymerase	Moloney leukemia virus DNA polymerase V (pmol of dGMP incorporated)	ALL polymerase T	Terminal transferase
Poly(C)·oligo(dG)	140	5.5	4.0	137
Poly(C) alone	<0.1	<0.4	0.4	—
Oligo(dG) alone	<0.1	<0.4	4.8	112

The data on AMV polymerase are taken from ref. 20. Moloney leukemia virus DNA polymerase V refers to the viral-specific polymerase recovered from virus-infected mouse-embryo bone-marrow fibroblasts processed in a manner identical to the ALL cells. Reaction mixtures of polymerase V and ALL polymerase T contained 50 μl of peak phosphocellulose fractions per reaction mix. Terminal transferase was prepared from calf thymus by Dr. F. N. Hayes of Los Alamos Scientific Laboratory, and was a kind gift of Dr. T. Kornberg.

We occasionally observe preparations that produce such "double peaks" for unknown reasons.) The second enzyme detected with poly(da-dT) was DNA polymerase N, eluting at 0.45 M. With poly(A)·oligo(dT) as template·primer, the third normal polymerase, DNA polymerase A, was evident at 0.3 M KCl (Fig. 1*B*). This enzyme eluted on the trailing edge of the DNA polymerase C peak, as it does in all other human cell preparations we have investigated. It is separable from DNA polymerase C by use of other fractionation methods (6).

Along with the three normal DNA polymerases, there was present, in the ALL cell extracts, a fourth activity that synthesized poly(dG) when provided with poly(C)·oligo(dG), and which eluted at 0.33 M KCl, a different salt concentration from that of any of the other enzymes (Fig. 1*C*). We call this enzyme DNA polymerase T. The demonstrated specificity of poly(C)·oligo(dG) as a template for the RNA-directed DNA polymerase of RNA tumor viruses (ref. 20; McCaffrey and Baltimore, unpublished results) led us to suspect that the fourth enzyme might be related to RNA-directed DNA polymerase. One simple experiment, however, disabused us of this notion.

The need for both the poly(C) template and the oligo(dG) primer to direct poly(dG) synthesis was tested for DNA polymerase T and for a series of other DNA polymerases (Table 1). The virion DNA polymerase of avian myeloblastosis virus (20) and the virus-specific DNA polymerase of Moloney murine leukemia virus-infected cells required the presence of both poly(C) and oligo(dG) in order to catalyze poly(dG) synthesis. DNA polymerase T, however, synthesized poly(dG) as well in the absence of poly(C) as in its presence. Polymerase T was, therefore, different from RNA-directed DNA polymerase; its properties were like those of an enzyme called terminal deoxynucleotidyl transferase (terminal transferase), which has been studied extensively by Bollum and his colleagues (1, 21). A sample of authentic (calf thymus) terminal transferase could be shown to synthesize poly(dG) when supplied only with an oligo(dG) primer (Table 1).

Polymerase T polymerized several different deoxyribonucleotides, and could utilize as primer several different oligomers, and even poly(dI) (Table 2). The enzyme functioned best in the presence of manganous ion, showing very little incorporation in the presence of magnesium (Table 3). The

TABLE 2. *Various oligomers and substrates as reactants with ALL polymerase T*

Primer	^{3}H-Labeled substrate	pmol of mononucleotide incorporated
Oligo(dG)	dGTP	5.0
	dATP	0.5
Oligo(dA)	dATP	5.1
Oligo(dT)	dTTP	0.6
	dGTP	11
Poly(dI)	dGTP	2

Standard assay conditions were used with 0.8 mM $MnCl_2$ and the following concentrations of reactants: oligo(dG), 4800 pmol; oligo(dA), 12,000 pmol; oligo(dT), 6800 pmol; poly(dI), 2400 pmol; and [^{3}H]substrates at concentrations and specific activities given in *Methods*. The peak phosphocellulose fraction from Fig. 1 served as enzyme source, 50 μl per reaction mix.

TABLE 3. *Requirements for dGMP incorporation by DNA polymerase T from ALL cells and human thymus*

Additions	ALL polymerase T (pmol of dGMP incorporated)	Thymus polymerase T (pmol of dGMP incorporated)
Oligo(dG); $MnCl_2$	13.5	5.5
Oligo(dG); $Mg(acetate)_2$	<0.5	<0.5
Oligo(dT); $MnCl_2$	15.5	6.5
None; $MnCl_2$	0.25	0.25

Standard reaction conditions were used with 2700 pmol of oligo(dG) or 6000 pmol of oligo(dT) and 1780 pmol of [^{3}H]dGTP, 2000 cpm/pmol. Either 6 mM $Mg(acetate)_2$ or 0.8 mM $MnCl_2$ was used. Peak phosphocellulose fractions from Figs. 1 and 2 were used as enzyme source, 50 μl per reaction mix.

phosphocellulose column eluate could be assayed for polymerase T with just an oligomer as primer and a single deoxynucleoside triphosphate as substrate (Fig. 1*D*).

Comparison with human thymus

Terminal transferase was originally purified from calf thymus, and has been demonstrated in the thymus of numerous animal species (9). In order to compare the cells from the ALL patient with human thymocytes, a specimen of human thymus was processed by the procedures used for the ALL cells. The elution profile of DNA polymerase activities from human thymus was very similar to the profile of the ALL cells (Fig. 2). The three normal enzymes were evident, plus a peak of activity that eluted at the same position as the ALL polymerase T and that synthesized poly(dG) when provided with oligo(dG) and dGTP. Like the enzyme from ALL cells, the thymic polymerase T was much more active with Mn^{++} than Mg^{++} and could polymerize dGMP when provided with an oligo(dT) primer (Table 3).

Assay of other human cells

We have assayed the DNA polymerases of several human cells using the same procedures as were used for the ALL cells.

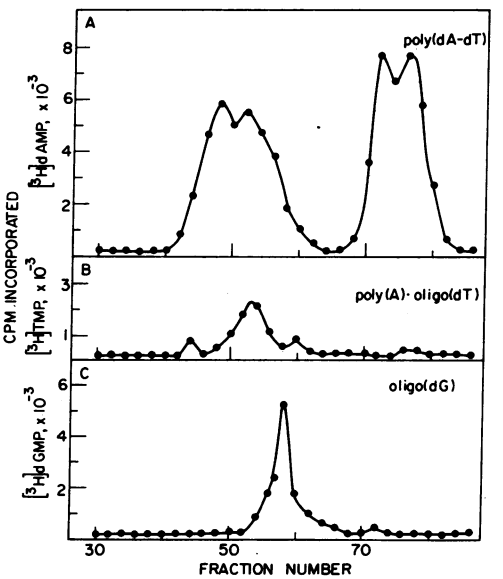

FIG. 2. DNA polymerases of normal human infant thymus.

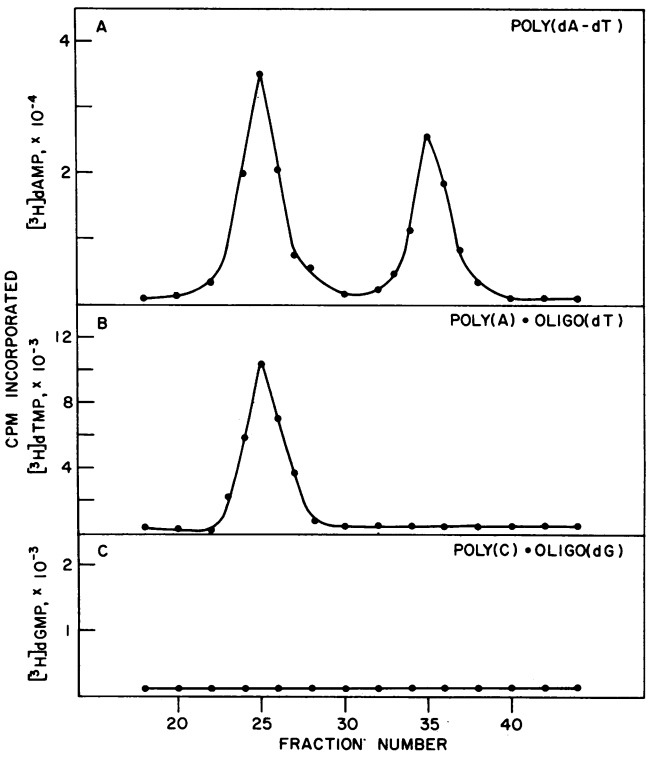

FIG. 3. DNA polymerases of HeLa cells.

Poly(dA-dT) was used to detect DNA polymerases N and C, poly(A)·oligo(dG) was used to detect polymerase A, and poly(C)·oligo(dG) was used to assay for the occurrence of either polymerase T or an enzyme with the properties of RNA-directed DNA polymerase.

Three cultured human cell lines were examined. HeLa cells, a line derived from a cervical carcinoma, had all three of the normal DNA polymerases, but no activity that could synthesize poly(dG) in the presence of poly(C)·oligo(dG) (Fig. 3). Similarly, a preparation of cultured human lymphocytes originally derived from a patient with mononucleosis had only the three normal enzymes (Fig. 4). A second line of cultured lymphocytes, initiated from a patient with acute lymphoblastic leukemia over 7 years ago, had a profile of DNA polymerases almost identical to that in Fig. 4, with no detectable stimulation of poly(dG) synthesis by poly(C)·oligo(dG). The relation of such long-term cultured cells to the original disease is not clear; these data are presented as controls indicating that growing cells contain no detectable enzyme related to DNA polymerase T.

Two preparations of neoplastic cells derived directly from patients were also assayed (Fig. 5 and 6). Both of these, one from a leukemic lymphosarcoma and the other from an acute myeloblastic leukemia, had a very low content of DNA polymerase C, presumably reflecting their nongrowing state. They also had very little DNA polymerase A activity (not shown). DNA polymerase N, however, was quite evident. No enzyme responding to poly(C)·oligo(dG) was evident in either extract, indicating that not all hematologic neoplasms contain a DNA polymerase T.

DISCUSSION

These data demonstrate that in at least one ALL patient, the leukemic cells contain a DNA polymerase not found in most other human cells. Among those cells that lack the activity are HeLa cells, cultured lymphocyte cell lines, and cells of patients with lymphosarcoma or myeloblastic leukemia. The only cells in which we have detected a similar enzyme are thymocytes. Sarngadharan *et al.* (22) have reported the occurrence of a new DNA polymerase in a *particulate* fraction from ALL cells. Their enzyme, which they consider to be similar to the RNA-directed DNA polymerase of RNA tumor viruses, might not be solubilized by the procedures we have used; thus, our data are possibly not comparable to their data. However, we have not found any activity similar to RNA-directed DNA polymerase in any of the human cells we have tested.

One implication of these data is that the ALL cells arose

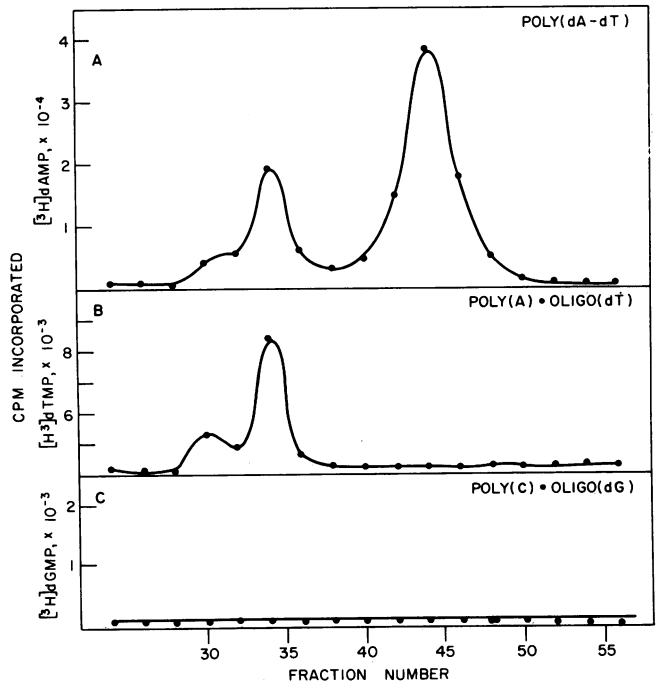

FIG. 4. DNA polymerases of L33-6-1-19 cultured human lymphocytes.

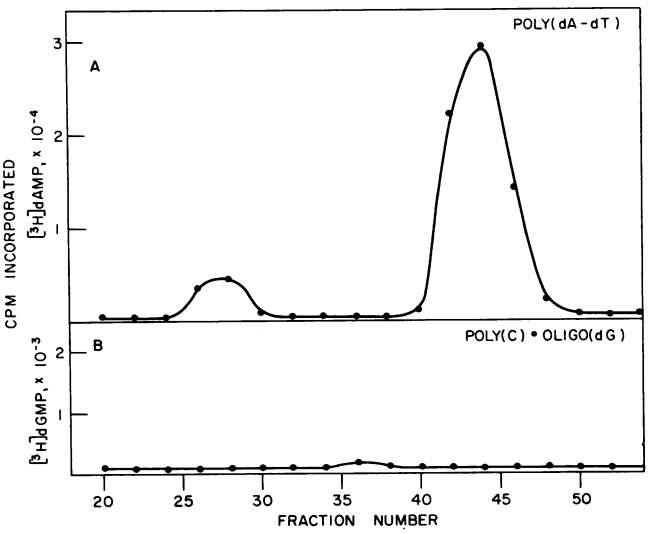

FIG. 5. DNA polymerases from the cells of a leukemic lymphosarcoma.

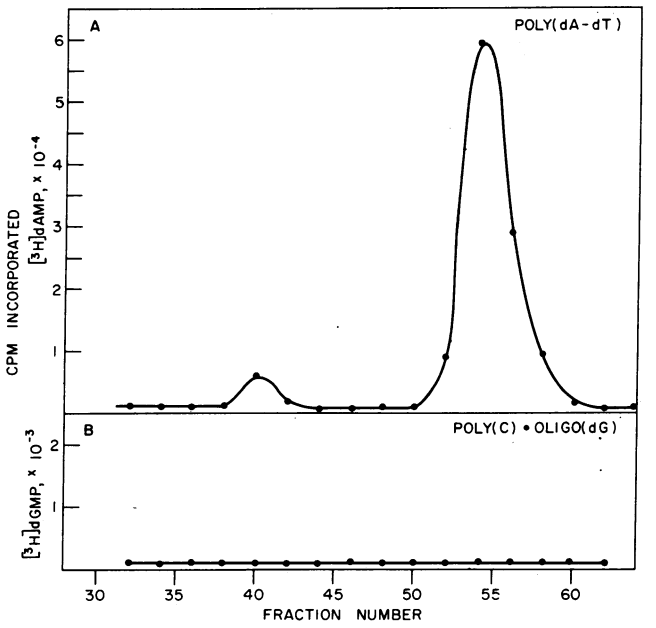

FIG. 6. DNA polymerases from the cells of an acute myeloblastic leukemia.

from thymocytes, and that assay of DNA polymerases can provide a marker for whether a given leukemia is of thymus or bone-marrow (bursal) origin. The study of Chang (9) showed that of many tissues assayed, only thymic cells had terminal transferase. Included among her negative cells were chicken cells from the bursa of Fabricius and mammalian cells from bone marrow, spleen, lymph nodes, circulating lymphocytes, liver, and lungs. The enzyme thus appears to be a marker for true thymocytes and not for "T-cells" (thymus-derived lymphocytes), since the circulating lymphoid cells, which include T-cells, show no activity. The ALL cells we have examined, therefore, appear to be thymic cells blocked very early in their differentiation.

Wilson and Nossal (23) have emphasized the diagnostic and therapeutic importance of distinguishing between B- and T-cell leukemias. They examined one case of ALL that was apparently of T-cell (or thymic) origin, because the cells lacked detectable surface immunoglobulin. By contrast, in their three cases of chronic lymphatic leukemia, surface immunoglobulin was evident on the cells, suggesting a B-cell origin. A thymic origin of some acute lymphoblastic leukemias is also suggested by the recent results of Minowada *et al.* (24).

We plan to analyze further cases of ALL and of other leukemias in order to determine if most ALL cases have DNA polymerase T, and whether this properly differentiates ALL, or maybe only childhood ALL, from other forms of lymphatic leukemia. The patient we have examined here has responded to conventional chemotherapy with remission, but not enough cells are now available to determine whether her remission lymphocytes still contain polymerase T. We hope to be able to assess whether loss of polymerase T is associated with remission.

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